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The CAR agonist TCPOBOP inhibits lipogenesis and promotes fibrosis in the mammary gland of adolescent female mice

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ABSTRACT

Constitutive androstane receptor (CAR) is a nuclear receptor that not only regulates drug-metabolizing enzymes but also influences energy metabolism. TC, 1, 4-bis [2-(3, 5-dichloropyridyloxy)] benzene (TCPOBOP) has been shown to inhibit lipogenesis in the liver and adipose tissues. The mammary gland is mainly composed of fat pads and duct systems in adolescent female mice. Here, activation of CAR by TC reduces the mammary gland weight, blocks lipid accumulation by inhibiting lipogenesis and gluconeogenesis, and accelerates collagen formation and fibrosis in the mammary fat pad of adolescent female mice. This information provides a reference for CAR activation, which may affect mammary gland development in adolescent females.

1. Introduction

Constitutive androstane receptor (CAR), also known as nuclear receptor subfamily 1, group I, member 3 (NR1I3), is a sensor and detoxifier of both endobiotic and xenobiotic substances (Baes et al., 1994; Yan et al., 2015). According to multiple published studies, this receptor plays roles in regulating drug-metabolizing enzymes, transporters and energy metabolism. CAR activation up-regulates the expression of target genes of the phenobarbital response element (PBRE) to prevent hepatotoxicity by hydroxylating, conjugating, and eliminating potentially harmful molecules (Wei et al., 2000; Gao and Xie, 2012; Yan and Xie, 2016). Recently, CAR has been reported to be a potential therapeutic target for the treatment of several metabolic diseases, including atherosclerosis (Sberna et al., 2011a; Sberna et al., 2011b), obesity (Gao et al., 2009), fatty liver disease (Dong et al., 2009) and diabetes (Dong et al., 2009; Gao et al., 2009), by balancing endogenous homeostasis of components such as cholesterol, bile acids, bilirubin and glucose.

TC, 1, 4-Bis [2-(3, 5-dichloropyridyloxy)] benzene, (TCPOBOP), which was initially isolated as a pesticide contaminant, is the most potent mouse CAR agonist and is well known to induce liver hypertrophy and hyperplasia in mice (Tzameli et al., 2000; Wei et al., 2000). TC also activates the human Pregnane X receptor (PXR) and is the most potent known member of the phenobarbital-like class of cytochrome P450 (CYP)-inducing agents (Smith et al., 1993). TC-induced CAR activation has been shown to improve insulin sensitivity, inhibit fat accumulation and ameliorate hepatic steatosis in both diet-induced obese and leptin-deficient (ob/ob) mice (Dong et al., 2009; Gao et al., 2009), activated CAR also reversed cholesterol transport in low-density lipoprotein receptor-deficient ($Ldlr^{-/-}$) and apolipoprotein E-deficient ($ApoE^{-/-}$) mice (Sberna et al., 2011a; Sberna et al., 2011b).

Treatment with TC induces CAR activation and influences lipid biosynthesis by targeting downstream lipogenic genes in the liver and adipose tissue (Yan et al., 2015). The mammary gland is an exocrine gland in mammals and is composed of fat pads formed by adipocytes and ductal systems (Brisken and Ataca, 2015). However, no experimental studies have evaluated the effects of TC on lipid metabolism in the mammary gland. Therefore, researchers have not determined whether TC-mediated CAR activation influences the development of the mammary gland. The aim of the present study was to investigate the effects of TC-induced CAR activation on lipid metabolism and fibrosis in the mammary gland of adolescent female mice. TC, the CAR agonist, reduced the mammary gland weight, decreased fat accumulation by

Abbreviations: ACC, acetyl-CoA carboxylase; α-SMA, alpha-smooth muscle actin; CAR, constitutive androstane receptor; FAS, fatty acid synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; G6pase, glucose-6-phosphatase; Gon-WAT, gonadal white adipose tissue; H&E, hematoxylin and eosin; IGF1, insulin-like growth factor 1; PBRE, phenobarbital response element; PAI-1, plasminogen activator inhibitor-1; PEPCK, phosphoenolpyruvate carboxykinase; PGC1α, peroxisome proliferator activated receptor gamma coactivator 1α; PPARα, peroxisome proliferator-activated receptor α; PXR, pregnane X receptor; SCD1, stearoyl-CoA desaturase 1; SREBP-1c, sterol regulatory element-binding transcription factor 1c; TC, TCPOBOP; XRE, xenobiotic response element

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inhibiting lipogenesis and gluconeogenesis, and promoted collagen formation and fibrosis in the mammary glands of adolescent female mice.

2. Materials and methods

2.1. Materials

TCPOBOP (T1442) and Oil Red O were purchased from Sigma-Aldrich (St Louis, Missouri, USA). TCPOBOP was dissolved in DMSO initially, and dissolved in saline configured to 0.05 mg/mL when used. Three-week-old female C57BL/6J mice were obtained from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). BODIPY® 493/503 (D3922) was purchased from Thermo Fisher Scientific (Shanghai, China).

2.2. Design of animal experiments

Three-week-old female C57BL/6J mice were randomly assigned to four groups (n = 5), housed in a light- and climate- controlled room (12-h light/dark cycle) and fed normal chow diets. TC-treated mice received intraperitoneal injections of TCPOBOP (0.5 mg/kg) twice per week for 2 or 5 weeks, and the vehicle-treated mice were injected with DMSO. Mice were euthanized with CO^2 at the indicated times. The mammary gland (fourth pairs), liver, and gonadal white adipose tissue (Gon-WAT) were carefully collected and weighted after the mice were sacrificed, and the samples were stored at −80 °C until further analysis. The experimental procedures were performed according to the guidelines of Ethics and Animal Welfare Committee College of Life Science Beijing Normal University, which approved the study (Approval No. CLS-EAW-2015-006); these guidelines conform to the US National Institutes of Health guidelines.

2.3. Histological analysis

Mammary glands, adipose and liver tissues were fixed with 4% formaldehyde. For paraffin sectioning, tissues were embedded in paraffin, cut into 5 μm sections and stained with Hematoxylin and Eosin (H &E), Masson's trichrome, and Sirus red staining using standard procedures (Altamirano et al., 2017; Wang et al., 2017; Xu et al., 2017a). Adipocyte sizes were analyzed using Image Pro Plus software. For frozen sections, tissues were embedded in OCT compound and sectioned into 8 μm sections. Frozen liver sections were stained with Oil Red O using a previously described method to observe lipid droplets (Xu et al., 2016). For immunofluorescence staining, frozen sections of the mammary glands and adipose tissue were incubated with a Cy3-conjugated α-smooth muscle actin antibody (1:400; Sigma-Aldrich) and then stained with BODIPY.

2.4. Gene and protein expression analysis

Total RNA was extracted from tissue using an RNAprep Pure kit from Tiangen Biotech Co., Ltd. (Beijing, China). For the real-time PCR analysis, reverse transcription was performed with oligdT-18 primers and M-MLV transcriptase from Promega (Madison, USA). SYBR Green qPCR SuperMix (TransGen Biotech, Beijing, China) was used to perform the reactions on an ABI 7500 real-time PCR system, according to the manufacturer's instructions and our previous study (Xu et al., 2017b). The primer sequences used are listed in Table 1. Western blotting analysis was performed used the standard process. The membranes were incubated with primary antibodies against fatty acid synthase (FAS, 1:1000; Santa Cruz), stearoyl-CoA desaturase-1 (SCD-1, 1:1000; Santa Cruz), sterol regulatory element-binding transcription factor 1c (SREBP-1c, 1:1000; Santa Cruz) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:2000; Santa Cruz), followed by the appropriate secondary antibodies. The intensity of the protein bands was quantified using ImageJ. Data were normalized to GAPDH levels.

Table 1
Primer sequences used for real-time PCR.

Target gene	Sequence(5′-3′)	Product length (bp)
ACC	F: GGACAGACTGATCGCAGAGA R: TGGAGAGCCCCACACACA	75
CYP2b10	F: AAGGAGAAGTCCAACCAGCA R: CTCTGCAACATGGGGGTACT	147
FAS	F: CCCTTGATGAAGAGGGATCA R: ACTCCACAGGTGGGAACAAG	115
G6pase	F: TCTGCCCCAGGAATCAAAAAT R: TGGGCAAAATGGCAAGGA	77
PAI-1	F: GTCTTTCCGACCAAGAGCAG R: ATCACTTGGCCCATGAAGAG	208
PEPCK	F: AGGAGGAGTACGGGCAGTTG R: CTTCAGCTTGCGGATGACA	62
PGC1α	F: GGAGCCGTGACCACTGACA R: TGGTTTGCTGCATGGTTCTG	176
PPARα	F: ATGCCAGTACTGCCGTTTTC R: TTGCCCAGAGATTTGAGGTC	168
SCD1	F: CCGGAGACCCCTTAGATCGA R: TAGCCTGTAAAAGATTTCTGCAAACC	89
SREBP-1c	F: AACCAGAAGCTCAAGCAGGA R: TCATGCCCTCCATAGACACA	141
GAPDH	F: GTCGTGGATCTGACGTGCC R: TGCCTGCTTCACCACCTTCT	72

2.5. Biochemical analysis

Liver lipid concentrations were measured using the commercially kits, as previously described (Xu et al., 2016). The liver triglyceride assay kit (E1013) and total cholesterol assay kit (E1015) were obtained from Applygen Technologies Co., Ltd. (Beijing, China).

2.6. Statistical analysis

The results are presented as the means ± SEM. Statistical analyses were performed using SPSS version 20.0 (IBM Corp). Differences between groups were analyzed using Student's *t*-test. Statistical significance was set to $p < 0.05$.

3. Results

3.1. TC-induced CAR activation reduced the mammary gland weight

Three-week-old female C57BL/6J mice were treated with vehicle (DMSO) or the CAR agonist TC (0.5 mg/kg, twice a week) for 2 or 5 weeks to determine the effect of TC on the mammary gland (Fig. 1A). As shown in Fig. 1B, TC had almost no effect on body weight. As expected, the expression of CYP2b10, a CAR target gene, was robustly elevated in the TC-treated mice (Fig. S1). Consistent with the findings of a previous study using an obese male mouse model (Gao et al., 2009), TC-induced CAR activation markedly enlarged the liver and inhibited fat accumulation in Gon-WAT (Fig. 1D and E). The mammary gland (breast) is mainly composed of fat pads. Fig. 1C showed a representative pair of mammary glands from a mouse that had been treated with the drug for 5 weeks, at which time the difference in the mammary gland weight between the TC-treated group and vehicle group was completely represented by the tissue/body weight ratio (Fig. 1F).

3.2. TC reduced fat accumulation in the liver, mammary fat pad and Gon-WAT in vivo

Activation of the CAR nuclear receptor ameliorates fatty liver disease in ob/ob mice and HFD-fed mice (Dong et al., 2009). In our normal control diet-fed mice, Oil Red O staining and the liver lipid content analysis indicated that TC also effectively inhibited lipid accumulation